STUDIES IN PHAGOCYTOSIS.*

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INTRODUCTION.

OUR knowlege of the finer mechanisms in the reactions of various infections is as yet in the beginning of its development. This is true especially of such common, clinically and anatomically well-understood infections as those caused by staphylococci and streptococci, pneumonia with its pneumococcemia, and typhoid fever with its bacillemia. The mechanisms, for instance, by means of which the typhoid bacillus overcomes the strong bacteriolytic power of normal human blood to which it seems very sensitive, and thus succeeds in establishing the typhoid infection, are certainly not fully understood. In our previous article on "The Antilytic Action of Salt Solutions and Other Substances"[1] we described certain experiments the results of which seemed to indicate "that in typhoid infections not only is complement used up, in the course of bacteriolysis, which no doubt is going on, but that a certain amount is also neutralized by the soluble products of disintegration of typhoid bacilli." Hence neutralization of complements may play an important rôle in the establishment of some infections in which we have reason to believe, as in the case of typhoid fever, that the bacteriolytic power of the blood is one of the most important means of defense and eventually also of recovery. In the case of streptococcus, staphylococcus, and pneumococcus infections, however, the situation is different because of the absence from the plasma of human and also of other kinds of blood of free bacteriolytic amboceptors with suitable complements for these organisms (with the possible exception in a limited degree of the staphylococcus). For this reason the extensive investigations of the last few years into the mechanisms of bacteriolysis and hemolysis by the serum of normal and immune animals have not afforded so promptly as at

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[1] *Jour. of Infect. Dis.*, 1904, 1, pp. 379-403.

first expected the desired basis for penetrating studies in the genesis and cure of these important infections in which general invasion of the blood is so prominent a feature. For these and other reasons of a more positive character we are now in the midst of a significant revival of interest in the relations of the leucocytes and of phagocytosis to the organisms concerned in these infections.* But we do not consider it necessary at this time to enter into any detailed discussion of the importance and scope in these and other infections of phagocytosis, the knowledge of which we owe largely to Metchnikoff and his pupils. Suffice it to point out that the recent work of Wright and Douglas concerning the action of normal plasma (and serum) in phagocytosis has opened the door for a deeper penetration into the nature of the process and the manner in which it may be modified in various directions.

THE OPSONINS OF WRIGHT AND DOUGLAS.

While many investigators have noted that the fluids of the body influence phagocytosis, especially, it was thought, by direct stimulation of the phagocytes, Wright and Douglas[1] were the first to show that phagocytosis by human leucocytes of various bacteria (S. pyogenes albus, M. melitensis, D. pneumoniæ, B. pestis, B. coli, B. dysenteriæ, B. typhosus, B. choleræ asiaticæ, B. anthracis, B. tuberculosis[2]) is directly dependent upon certain substances in the plasma (and in the serum) which they call opsonins.[3]

They concluded that these substances are taken up by the bacteria which then become susceptible to phagocytosis because bacteria digested in serum heated to 60 or 65° C. for 10 to 15 minutes are not taken up by leucocytes, whereas bacteria digested in normal serum and then heated to 60° C. for 10 minutes are taken up readily. The opsonic power of serum is removed by digestion with dead bacteria and by the addition of Daboia venom; it disappears gradually from standing serum, and, as just indicated, it is destroyed by heat to 60 or 65° C.

*In connection with this see G. F. RUEDIGER, "Mechanism of Streptococcus Infection," *Trans. of Section on Path. and Phys. of Am. Med. Assoc.*, 1904, p. 397.

[1] *Proc. of the Roy. Soc.*, 1903, 72, p. 357 and 1904, 73, p. 128.

[2] *The Lancet*, October 22, 1904, 2, p. 1138.

[3] From the Latin *obsono* or *opsono*, "I cater for," "I prepare food for."

It occurred to us that it might prove of interest to study the action of temperature and of different chemical substances upon the bodies concerned in phagocytosis. It was hoped that in this way it might prove possible more closely to analyze the phenomenon itself as well as to learn something of the ways in which the phagocytic power may be modified in those infections in which phagocytosis is thought to be an important means of defense.

TECHNIQUE OF THE STUDY OF PHAGOCYTOSIS *IN VITRO*.

Within certain more or less obvious limitations the phenomenon of phagocytosis lends itself readily to studies *in vitro*. Wright and Douglas employed with satisfaction Leishman's method[1] of measuring the phagocytic power of the leucocytes in their experiments. Equal parts of blood or other fluid containing leucocytes and bacterial suspensions were mixed and exposed to 37° C. for 15 minutes when smears were stained with Leishman's modification of Romanowsky's method and counts made of the bacteria in a certain number of phagocytes. In this way they obtained averages of the numbers of bacteria taken up by the cells.

In our experiments we have followed the same general technical method. In the main we have used defibrinated blood, but in the case of blood of the rabbit and of the guinea pig it often becomes necessary to add leucocytes from pleural exudate because the defibrinated blood of these animals contains so few polymorphonuclear leucocytes. In certain experiments suspensions of washed leucocytes have been made in serum and in 0.85 % NaCl solution. In general a definite quantity of blood or of leucocytic suspension or of a mixture of these and various salt solutions, and an equal quantity of bacterial suspension, are introduced by means of finely graduated pipettes into small test-tubes which are then incubated at 37° C. for one hour when smears are made and stained with Leishman's stain. The number of bacteria in at least 20 leucocytes are counted in each preparation and the averages of the counts so obtained are shown by the figures in the tables.

[1] *Brit. Med. Jour.*, Jan. 11, 1902, 1, p. 73.

PRELIMINARY EXPERIMENTS.

The results of our preliminary experiments with few exceptions corroborate fully the principal observations of Wright and Douglas upon the influence of human serum on phagocytosis of many bacteria by human polymorphonuclear neutrophile leucocytes. We have found further that similar conditions obtain in the case of phagocytosis by the leucocytes of various animals such as the guinea pig, rabbit, dog, goat, white rat, and horse. The leucocytes of the dog and guinea pig, like those of man, lend themselves especially well to the study of the mechanism of phagocytosis.

Table I shows the phagocytic power, numerically expressed, of the polymorphonuclear leucocytes in the defibrinated blood of these animals under the conditions of our experiments. The

TABLE I.

PHAGOCYTOSIS BY LEUCOCYTES IN DEFIBRINATED BLOOD.

Organisms	Human leucocytes	Guinea pig leucocytes	Rabbit leucocytes	Dog leucocytes	Goat leucocytes	White rat leucocytes	Horse leucocytes
Streptococcus 300[1]	24	50	20	50	30	40	50
Streptoc. 381 P [2]...	23	0	0.5	25	..	5	..
Streptococcus 104[3]	20	2	0	13	0	0	4
Pneumococcus	0	0	0	0	0	..	0.1
Staphyloc. aureus .	27	50	12	13	12	9	..
B. typhosus.....	5	6.5	5	11	15	13	7.5
Micrococcus X[4]....	1.5	4	3	7.6	..	..	28

[1] Isolated from the heart's blood of a fatal case of scarlet fever; non-virulent for rabbits and guinea pigs.

[2] Isolated from the pericardium of a fatal case of scarlet fever; kills guinea pigs and rabbits in doses (ascites broth cultures) of 0.5 % of body-weight.

[3] Isolated from abscess in guinea pig; kills guinea pigs and rabbits in doses of one c.c. or less of 24-hour ascites broth or serum broth cultures.

[4] An unidentified micrococcus non-virulent for rabbits.

absence of phagocytosis in the case of some of the organisms cannot be discussed at this time further than to say that we are now engaged in further studies, the results of which we hope may throw some light upon the problems thus presented. In the case of organisms with such variable physiological properties as the pneumococcus, we believe that the results shown in Table I should not be applied offhand to other strains, especially in view of the contrary results of Wright and Douglas.

On account of the pronounced variation in the susceptibility of various organisms to phagocytosis, it would seem not unlikely that we have here another means of differentiation that may prove useful.

In the case of anthrax bacilli it is difficult if not impossible to count the number of bacilli taken up by the leucocytes. The preparations from the experiments made in the manner outlined with normal human, dog, and goat leucocytes show, however, that practically every polymorphonuclear leucocyte is actively engaged in phagocytosis.

That phagocytosis under these circumstances depends on the action of something in the serum on the bacteria is shown (1) by the absence of phagocytosis on the part of washed leucocytes suspended in salt solution and mixed with untreated bacteria (a fact emphasized by Wright and Douglas, and observed by us in a large number of instances without a single exception), and (2) by good phagocytosis on the part of similarly treated leucocytes mixed with bacteria first digested with normal serum, then washed and suspended in salt solution. Hereafter we shall speak of bacteria so treated as sensitized bacteria. That leucocytes have a special affinity for sensitized bacteria is seen from the fact that in the presence of such bacteria and carmin granules the leucocytes show a marked preference, so to speak, for the bacteria. As is shown in the following Table II various serums may serve to sensitize a non-virulent streptococcus for phagocytosis by human leucocytes:

TABLE II.

PHAGOCYTOSIS BY HUMAN LEUCOCYTES OF BACTERIA SENSITIZED WITH ALIEN SERA.

Human leucocytes	(defibrinated blood)	+	Staphylococcus aureus	22.0
" "	washed, in NaCl solution	+	" "	1.2
" "	" " " "	+	" " treated with human serum	10.0
" "	(defibrinated blood)	+	Streptococcus 300	22.0
" "	washed, in NaCl solution	+	" "	1.0
" "	" " " "	+	" " treated with human serum	14.0
" "	" " " "	+	" " " " guinea pig serum	12.0
" "	" " " "	+	" " " " rabbit serum	14.0

NOTE.—We have found that in order to obtain the same degree of phagocytosis in different experiments it is important that approximately the same number of bacteria be present. In the experiment above many cocci were undoubtedly lost during the process of sensitization and washing.

The phagocytic power of the leucocytes in the pleural aleuronat exudates of the dog, guinea pig, and rabbit is materially increased

after centrifugating and resuspension in serum or defibrinated blood. This indicates that the fluid of the blood contains more opsonin than the exudate. Streptococcus 300 is readily sensitized by normal horse serum for phagosytosis by human leucocytes.

We have not been able to sensitize with human serum a virulent streptococcus so that it is taken up by washed guinea pig leucocytes. This streptococcus is readily taken up by normal human leucocytes, but not by the leucocytes of normal guinea pigs. The interesting problems presented by this observation are reserved for further consideration in connection with the study of phagocytosis by leucocytes of immunized animals. Of course, the possibility of different opsonins even in the same species must be considered.

At low temperatures—1 to 4° C.—bacteria are not sensitized so rapidly by far as at 35 to 37° C.

Table III also shows the influence of serum upon phagocytosis in a striking manner. In this experiment each tube contained 0.3 c.c. of suspension of washed guinea pig leucocytes and falling quantities of guinea pig serum, enough 0.85% NaCl solution being added to make 0.6 c.c. in each case, and 0.5 c.c. of a suspension of Streptococcus 300.

TABLE III.

THE QUANTITATIVE EFFECT OF SERUM ON PHAGOCYTOSIS.

Quantity of Serum	Number of Cocci Taken up
0.2 c.c.	23.4
0.1 "	19.2
0.05 "	14.0
0.025 "	7.5
0.0125 "	2.2
0.006 "	1.5
0.003 "	0.7
0.000 "	0.0

THE EFFECTS OF TEMPERATURE ON OPSONINS IN NORMAL SERUM.

According to Wright and Douglas, bacteria digested with serum heated to 60 or 65° C. for 10 to 15 minutes are not taken up by leucocytes, whereas bacteria digested with normal serum and then heated to 60° C. for 10 minutes are taken up freely.

Our own experiments show that the power to sensitize streptococcus 300 for phagocytosis by washed corpuscles is lost on heating human, rabbit, and guinea pig sera to 54 to 56° C. and dog serum to 58 to 60° C. for 30 minutes. Low temperatures down to 46° C. materially lessen the power of all these sera to sensitize streptococcus 300 for phagocytosis by washed homologous leucocytes.

We have found that when cocci, once sensitized, are exposed for 30 minutes to the temperature at which the serum used for sensitization is inactivated, or preferably 3–4° higher, the cocci are not taken up to any extent either by washed leucocytes or by leucocytes in defibrinated blood (Table IV). Evidently the substance—opsonin—taken up from the serum by the receptors of the cocci becomes changed in such a manner that it not only no longer renders the cocci fit for phagocytosis, but actually prevents the cocci from taking up new opsonin.

Digestion of cocci in serum inactivated by heat does not interfere with their phagocytes by leucocytes in fresh serum, indicating that in the heated serum there no longer is opsonin capable of union with the leucocytes, and heating non-sensitized cocci to 56° C. and higher does not prevent them from becoming sensitized and taken up by leucocytes.

TABLE IV.

PHAGOCYTOSIS OF HEATED STREPTOCOCCI, SENSITIZED AND NON-SENSITIZED, BY HUMAN LEUCOCYTES.

A. Washed Leucocytes.	
Sensitized streptococci (300)	16
" " " heated to 58° C. 30 min.	4.2
" " " " " 60° " " "	2.5
Non-sensitized " "	0.1
B. Leucocytes in Defibrinated Blood.	
Sensitized streptococci (300) heated to 58° C. 30 min.	4.6
Non-sensitized " " " " " " " "	12.6
" " " " " 60° " " "	12.5
" " "	15

It appears as if opsonin, like toxins and complements, possesses two groups of molecules, one haptophore whereby it attaches itself to the bacterial receptors, and one, which may be called the opsoniferous group, whereby is effected in the bacterium some change, physical or chemical, that is necessary for phagocytosis. We may say that when sensitized cocci are heated, the

opsoniferous group is largely inactivated, but as the bacterial receptors remain occupied by the haptophore group, the bacteria are prevented from taking up new opsonin. In accord with Ehrlich's nomenclature, opsonin, the opsoniferous group of which is destroyed or inactivated may be termed opsonoid. So far we have not obtained indications that any amboceptor is concerned in the sensitization of bacteria for phagocytosis by the leucocytes of normal animals.

Whether opsonins under suitable conditions may give rise to the production by animal and bacterial cells of antiopsonins, and whether certain organisms, virulent and otherwise, are protected from phagocytosis by lack of suitable receptors or by the production of antiopsonins or by other means, singly or combined, are problems upon which work is now in progress.

It certainly is of great interest that Ehrlich's lateral chain theory should prove useful in making clear to us certain phases of the complex mechanism of phagocytosis, the physical and chemical laws of which are not wholly understood. The interdependence of the "humoral" and cellular forces in the reactions set up by bacteria receives here a striking demonstration of no little interest when we recall the conflicts between the humoral and cellular theories of immunity.

THE EFFECT OF SALT SOLUTIONS AND FORMALIN UPON PHAGOCYTOSIS BY LEUCOCYTES OF NORMAL ANIMALS.

For the purpose of studying the effect of salt solutions upon phagocytosis by leucocytes of normal animals a number of experiments have been made. These experiments the results of which are given in Tables V and VI were made as follows: Freshly drawn defibrinated blood was mixed with an equal quantity of $\frac{m}{8}$ solutions of the various salts used; whenever less of the $\frac{m}{8}$ solutions was added, the deficit was made up by means of 0.85 5 % NaCl solution. These mixtures were then placed at 37° C. for 30 to 60 minutes, when an equal quantity of suspension of 24-hour growths of the bacteria used was added and the tubes returned to the incubator for one hour more. Smears were made and the number of bacteria taken up by the leucocytes determined as described in the foregoing. The tables show that practically every

TABLE V.

THE ANTIPHAGOCYTIC ACTION OF $\frac{m}{8}$ SALT SOLUTIONS AND OF FORMALIN.

LEUCOCYTES	MICROBE	NaCl	$CaCl_2$		$BaCl_2$		$SrCl_2$		$MgCl_2$		K_2SO_4		$NaHCO_3$		$Na_3C_6H_5O_7$		$Na_2C_2O_4$		$K_4Fe(CN)_6$		FORMALIN		
																					1-2000	1-5000	
		0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.1
Human	Staph. aureus	26	16.5	17	1	20	18.6	23	10	18	13	12	13	15	12	12	12	17	2	16	3	11	18
Human	Strept. (300)	17	3.4	11	0	5.8	2	13	0	13	8	8.3	5.6	8.5	12.5	10.7	10	10	0	10	3	12	14
Human	B. typhosus	7.6	8	12	0	11	10	10.6	12.4	8.8	3.9	5.7	0	2.8	3 8	4	1.8	4.5	2.2	6.4	0	1.5	2.7
Dog	Staph. aureus	17	7.4	14	10	12.4	16	18	9.5	11	15	17	2	14	2.8	10	4	12	1	7	2	7	7.5
Dog	Strept. (300)	21	12	17.5	12	19	17.5	22	18	20	17	21	10	17	13.5	20	7	21	0	11.5	1	11	20
Dog	B. typhosus	12	6.7	7.5	3	4	5	7	2.5	7	3	4	1	4.6	1.5	5	3	3.6	0	5	1.5	6	6
Guinea pig	Staph. aureus	9	0	2	0	3	2	6	3	4.5	5	7	0	2.5	0	2	0	2	0	2	0	2	3
Guinea pig	Strept. (300)	33	13	18.6	3	11	13.5	18.5	20	27	26	28	15	22	2	11	2	9.5	0	4	0	0	6
Guinea pig	B. typhosus	6.5	0.5	3	0	1.5	2.5	·4	3.5	4.7	3.5	5	1.5	3	0	0	0	0	0	0	0	2	2

TABLE VI.

ANTIPHAGOCYTIC ACTION OF $\frac{m}{8}$ SALT SOLUTIONS AND OF FORMALIN UPON HUMAN LEUCOCYTES IN PRESENCE OF ANTHRAX BACILLI.

DEFIBRINATED HUMAN BLOOD + SALT SOLUTION + SUSPENSION OF ANTHRAX BACILLI	PHAGOCYTOSIS (50 Leucocytes Counted)	
	Phagocytosis	No Phagocytosis
Blood 0.3 c.c. + $\frac{m}{8}$ solution NaCl 0.3 c.c. + suspension 0.6 c.c.	50	0
" 0.3 " + " " $CaCl_2$ 0.3 " + " " "	0	50
" 0.4 " + " " " 0.2 " + " " "	20	30
" 0.3 " + " " $BaCl_2$ 0.3 " + " " "	0	50
" 0.4 " + " " " 0.2 " + " " "	3	47
" 0.3 " + " " $SrCl_2$ 0.3 " + " " "	0	50
" 0.4 " + " " " 0.2 " + " " "	43	7
" 0.3 " + " " $MgCl_2$ 0.3 " + " " "	13	37
" 0.4 " + " " " 0.2 " + " " "	25	25
" 0.3 " + " " K_2SO_4 0.3 " + " " "	46	4
" 0.4 " + " " " 0.2 " + " " "	50	0
" 0.3 " + " " $NaHCO_3$ 0.3 " + " " "	0	50
" 0.4 " + " " " 0.2 " + " " "	15	35
" 0.3 " + " " $Na_3C_6H_5O_7$ 0.3 " + " " "	2	48
" 0.4 " + " " " 0.2 " + " " "	10	40
" 0.3 " + " " $Na_2C_2O_4$ 0.3 " + " " "	0	50
" 0.4 " + " " " 0.2 " + " " "	25	25
" 0.3 " + " " $K_4Fe(CN)_6$ 0.3 " + " " "	3	49
" 0.4 " + " " " 0.2 " + " " "	26	24
Blood 0.3 c.c. + Formalin 1-2000 0.3 c.c. + suspension 0.6 c.c.	0	50
" 0.4 " + " " " 0.2 " + " " "	10	40

salt used, as well as formalin, materially reduces the amount of phagocytosis as compared with the amount obtained in the control experiment with 0.85 % ($\frac{m}{6.8}$) NaCl solution. In many cases there has been produced a complete suspension of phagocytosis, notably in the case of certain experiments with $BaCl_2$, $NaHCO_3$, $Na_2C_2O_4$, $Na_3C_6H_5O_7$, $K_4Fe(CN)_6$, and formalin. Attention must be called to the fact that it is rather difficult to count accurately typhoid bacilli situated within leucocytes; in many cases the bacilli seem to break into fragments each of which may be mistaken for a complete bacillus. In the case of anthrax bacilli (Table VI) no attempt has been made to count the bacilli within leucocytes, and for obvious reasons. We believe that in this instance the counts of leucocytes with reference to their being engaged in phagocytosis or not give an adequate idea of the influence of the salts studied, and of formalin, which is found to be about the same as that shown by Table V. $MgCl_2$ and K_2So_4 seem to have less antiphagocytic action than the other salts, occupying in this respect the same relative position as in our tables showing the antilytic action of salt solutions.[1]

MODE OF ACTION OF ANTIPHAGOCYTIC SALTS.

The question how these salts (and formalin) hinder phagocytosis, whether by their action on the leucocytes, the cocci, or the serum, is an interesting one. In order to throw some light upon the antiphagocytic mechanism we have made certain experiments the results of which appear to indicate that the serum is the principal point of attack. If the salts, in the dilutions used, had a direct toxic action on the leucocytes one would not expect any phagocytosis on the part of leucocytes that had been suspended for one to two hours in the salt solutions, then centrifugated out and resuspended in normal serum. The following experiment shows, however, that leucocytes digested for one and a half hours in $\frac{m}{8}$ solutions of calcium chloride, potassium ferrocyanide, and trisodium citrate and then transferred again to normal serum take up fully as many streptococci as those treated in the same manner with 0.85% solution of sodium chloride:

[1] HEKTOEN AND RUEDIGER, *loc. cit.*

Defibrinated guinea pig's blood, to which are added leucocytes from a pleural exudate, is centrifugated, the serum withdrawn and replaced by 0.85% NaCl solution; 0.5 c.c. of this suspension of corpuscles is added to four c.c. of solutions of NaCl, $CaCl_2$, $Na_3C_6H_5O_7$, and $K_4Fe(CN)_6$ respectively and the tubes placed at 37° C. for one and a half hours. The cells are now centrifugated out, washed once in NaCl solution and resuspended in 0.3 c.c. of guinea pig serum, making in all about 0.5 c.c. of suspension to which is added 0.5 c.c. of streptococcus (300) suspension. The tubes are again incubated one hour when stained preparations are made and the bacteria taken up by the leucocytes counted with the following average result for each leucocyte:

Leucocytes	treated	with	NaCl	solution	= 15.1
"	"	"	$CaCl_2$	"	= 11.8
"	"	"	$Na_3C_6H_5O_7$	"	= 16.8
"	"	"	$K_4Fe(CN)_6$	"	= 15.8

The average number of cocci within the leucocytes in the $CaCl_2$ tube is a little smaller than in those from the NaCl tube, but the difference is so small as to fall within experimental error.

Digestion of cocci for half an hour in salt solutions (NaCl, $CaCl_2$, $Na_2C_2O_4$, $K_4Fe(CN)_6$), and then removing them by centrifugating, and again suspending them in NaCl solution does not hinder their sensitization to the usual extent by dog serum.

The following experiment shows that $\frac{m}{8}$ solutions of various salts have a marked inhibitory effect on phagocytosis when added to defibrinated blood before adding bacteria (see also Tables V and VI). If, however, the bacteria added to the mixtures are previously treated with normal serum and washed in NaCl solution nearly the same number of organisms are taken up by leucocytes as in a mixture of defibrinated blood and bacterial suspension alone. This indicates again that the inhibitory salts have no toxic effect upon the leucocytes in the mixtures.

Two sets of tubes are prepared, each containing 0.3 c.c. of defibrinated human blood and 0.3 c.c. of NaCl, $CaCl_2$, $Na_3C_6H_5O_7$, $Na_2C_2O_4$, and $K_4Fe(CN)_6$ solutions respectively. The tubes are placed at 37° C. for one hour and then there is added to one set untreated streptococci (300), to the other set sensitized streptococci (*i. e.*, streptococci digested with human serum at 37° C. for 30 minutes, then washed and suspended in NaCl solution); the tubes are again incubated for one hour, when smears are made and the number of bacteria taken up counted. The result is shown in Table VII.

TABLE VII.

THE EFFECT OF SALT SOLUTIONS UPON PHAGOCYTOSIS OF NON-SENSITIZED AND SENSITIZED STREPTOCOCCI.

$\frac{m}{8}$ Salt solutions	0.3	Phagocytosis of Non-Sensitized Streptococci	Phagocytosis of Sensitized Streptococci
Defibrinated blood	0.3		
Streptococcal suspension	0.5		
NaCl		12	10
$CaCl_2$		0.5	10.8
$Na_3C_6H_5O_7$		1	9.7
$Na_2C_2O_4$		0.9	7.1
$K_4Fe(CN)_6$		2	7.7

As stated the results of this experiment practically demonstrate that the salts do not owe their antiphagocytic action to any direct toxic effect upon the leucocytes. On the contrary they indicate that the salts act upon the serum, *i. e.*, upon the opsonin which is prevented from so changing the cocci as to make their phagocytosis possible. This being the case it should not be possible to sensitize bacteria to any extent in mixtures of serum and antiphagocytic solutions. And experiment does show that bacteria digested for half an hour in mixtures of serum and $\frac{m}{8}$ solutions of $CaCl_2$, $Na_3C_6H_5O_7$, $K_4Fe(CN)_6$ (as well as in mixtures of serum and formalin—1:2000 in NaCl solution—and of serum and $\frac{m}{1}$ solutions of KCl and NaCl), and suspended in 0.85% NaCl solution are not taken up nearly so well by washed leucocytes as cocci that are sensitized in the usual way.

Human serum 0.2 c.c. and 0.4 c.c. of solutions of NaCl, $CaCl_2$, $Na_3C_6H_5O_7$, $K_4Fe(CN)_6$ respectively are mixed and placed at 37° C. for one-half hour when 0.5 c.c. of a thick suspension of streptococci (300) is added and the incubation continued for half an hour longer; the cocci are now centrifugated out, resuspended in NaCl solution 0.4 c.c. and 0.4 c.c. of a suspension of washed human leucocytes added to each tube. The tubes are again incubated at 37° C. for 45 minutes when smears are made and the bacteria taken up counted with the following result:

Washed leucocytes	+	cocci	treated	in	serum	and	NaCl	- - -	7.0
"	"	+ "	"	"	"	"	$CaCl_2$	- - -	0.8
"	"	+ "	"	"	"	"	$Na_3C_6H_5O_7$	- -	0.85
"	"	+ "	"	"	"	"	$K_4Fe(CN)_6$	- -	0.9
"	"	+ unsensitized cocci						- - - - - -	0.

We wish to state that the identical results cannot always be obtained with leucocytes and serum from different individuals and from different species, but in all our experiments there has been noted a more or less marked diminution in phagocytosis of cocci treated in serum-salt mixture as compared with the controls.

The same experiment as the previous one, but with anthrax bacilli, dog serum and dog leucocytes, human serum and human leucocytes, gave the results shown in Table VIII, 50 leucocytes being counted with reference to their being engaged or not in phagococytosis:

TABLE VIII.

PHAGOCYTOSIS OF ANTHRAX BACILLI TREATED IN MIXTURES OF SERUM AND $\frac{m}{8}$ SALT SOLUTIONS.

WASHED LEUCOCYTES + TREATED ANTHRAX BACILLI	PHAGOCYTOSIS (50 Leucocytes Counted)			
	Human Serum and Leucocytes		Dog Serum and Leucocytes	
	+	0	+	0
Washed leucocytes + bacilli treated in serum and NaCl.....	38	12	45	5
" " + " " " " " $CaCl_2$.....	1	49	17	33
" " + " " " " " $Na_3C_6H_5O_7$.	5	45	4	46
" " + " " " " " $Na_2C_2O_4$.....	1	49	0	50
" " + " " " " " $K_4Fe(CN)_6$.	0	50	1	49
" " + untreated bacilli	4	46	8	42

From the further fact that bacteria remaining unsensitized in serum-salt mixtures are readily sensitized in fresh serum we conclude that the antiphagocytic salt solutions neutralize or bind the opsonin in such a manner that it cannot act upon the bacteria to the full extent. There is always some phagocytosis in the mixtures of blood and salt solutions, and some cocci are sensitized in the serum-salt mixtures even when relatively large quantities of salt solutions are used. When sensitized cocci are added to the mixture of blood and salt solutions the number of cocci taken up by the leucocytes in the tubes containing $CaCl_2$, $Na_3C_6H_5O_7$, $K_4Fe(CN)_6$, etc., is somewhat smaller than that taken up in the tube containing NaCl. At first sight these facts appear somewhat puzzling, but they are easily understood if we assume that the compounds formed by the union of opsonin with salt, or one of the ions of the salt, and opsonin with bacteria are dissociated, that is, that the reactions are reversible. All of the opsonin in a mixture of salt and serum may have united with the salt, but when cocci are added to the mixture there is established an equilibrium between opsonin and salt on the one hand and opsonin and bacteria on the other and some bacteria will necessarily be sensitized. Similarly, if a mixture of blood and salt

contains a slight excess of salt and sensitized cocci are added to this mixture some of the bacteria will be deprived of their opsonin when equilibrium is established between opsonin and salt on the one hand and opsonin and bacteria on the other, and therefore the number of bacteria taken up by the leucocytes in such a mixture is smaller than might be expected.

SUMMARY.

In closing we wish to emphasize the following points:

1. Phagocytosis of many bacteria by the leucocytes of various normal animals, including man, is dependent upon the presence in the plasma of special substances designated by Wright and Douglas as opsonins.

2. The opsonins become attached to the bacteria which then for unknown reasons become susceptible to phagocytosis.

3. The opsonins in the blood of one species may sensitize bacteria for phagocytosis by the leucocytes of a different species.

4. Opsonins are thermolabile substances of a constitution analogous to that of toxins and complements in that they seem to have two groups, haptophore and opsoniferous; by heating sensitized bacteria the opsoniferous group appears to be destroyed, but the inactive opsonin (opsonoid) by saturating the receptors of the bacteria prevents further sensitization by fresh serum.

5. Like complements opsonins may be neutralized or bound by various salt solutions ($CaCl_2$, $BaCl_2$, $SrCl_2$, $MgCl_2$, K_2SO_4, $NaHCO_3$, $Na_3C_6H_5O_7$, $Na_2C_2O_4$, $K_4Fe(CN)_6$) and other substances, *e. g.*, formalin, so that they cannot act upon bacteria. Antiphagocytic action of this nature may be an important factor in the establishment and progress of various infections, especially those caused by streptococci, pneumococci and other microbes in the destruction of which phagocytosis is an important factor.